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Research Article

Bioremediation potential of *Bacillus cereus* against copper and other heavy metals

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Abstract

Aim: This study is aimed at designing a bioremediation model using microbes against Cu^{2+} pollution. **Methods And Results**: For this purpose, copper resistant bacteria were isolated from industrial areas and were characterized for its copper accumulating properties using various methods including copper accumulation, optimization, Antibiotic resistance, production of thiols, plasmid curing, SEM. The most potential strain was isolated based on its maximum tolerable capacity (MTC) of 600ppm copper accumulation. All assays were carried out under optimized culture conditions. Antibiotic resistant genes and metal resistant genes are said to get transferred together in the environment, hence both resistance were studied to confirm the hypothesis. Production of thiol compounds in response to copper toxicity seemed high. Plasmid curing confirmed the location of the copper resistant genes in the chromosomes. SEM study confirmed high copper accumulating capacity of the bacteria. **Conclusions:** The high copper accumulating property of the bacteria might be attributed to the highly contaminated nature of the sampling site. The level of accumulation and toleration can be enhanced by perfect optimization of all environmental and conditional parameters. On increase in the potency of the bacteria, it can be well recommended as a choice for its use in environmental applications.

Keywords: Bacillus, Bioremediation, SEM, Copper accumulation, plasmid curing**.**

Introduction

Copper ions pose a dual challenge to both eukaryotic and prokaryotic cells as these serve as a micronutrient for both kingdoms, but do however become toxic above optimum levels (Munson *et al*. 2000). In this regard, copper is an essential ion that is involved in some metabolic processes such as being a component (co-factor) of many metalloenzymes where it plays a role in the active sites of these enzymes (Harris 2000). The ability however of this metal to generate free radicals has contributed to its potential toxicity. As such, copper ions are capable of catalyzing harmful redox reactions which result in the oxidation of lipid membranes and damage to nucleic acids (Hoshino *et al*. 1999). Some bacteria have, in turn, developed detoxification systems to protect themselves from toxic concentrations of copper ions while still ensuring that these ions meet their nutritional requirements (Hoshino *et al*. 1999). These mechanisms vary from active efflux to sequestration, cell wall modification and bioprecipitation (Choudhury and Srivastava 2001). Such heavy metal resistant microorganisms are very useful in biotechnology for the remediation of metal contaminated environments and can also be used in the construction of biomarkers for the detection of metals.

Some bacteria have evolved a set of mechanisms that control and respond to the uptake and accumulation of heavy metals. Possible interactions between toxic metals and bacteria include (a) production and secretion of organic acids, polysaccharides, melanins, or proteins and subsequent binding/complexation and

precipitation of metal ions (b) metal binding to cell walls (c) transport of metal cations (d) chemical transformation of metals (e) organellar compartmentation and (f) synthesis of thiol-containing compounds, such as the nonproteinaceous glutathione and phytochelatins and the metallothionein proteins of families 8–13 which can sequester metal ions (Guimaraes et al., 2005)

Biosorption, the process of passive binding by dead or living biomass, represents a potentially cost-effective method to remove heavy metals from industrial wastewaters and could be employed most effectively in a concentration range below 100 mg/L. Algae, bacteria, fungi and yeasts have proved to be potential metal biosorbents (Xue Song Wang 2009).

This study deals with designing a bioremediation model using microbes against Cu^{2+} pollution. For this purpose, copper resistant bacteria were isolated from industrial areas, and their characteristics were determined to exploit them for bioremediation of Cu^{2+} contaminated toxic wastewaters.

Materials and Methods

Collection of samples

In order to isolate bacteria that are able to tolerate high copper concentrations, heavily contaminated copper producing industrial areas around Tuticorin coast was selected. Soil and water samples were collected in sterile screw cap tubes and were transferred immediately to laboratory conditions in ice bag.

Isolation of copper resistant bacteria

The soil and water samples were enriched for 48h in nutrient broth media along with a pinch of Nystatin to prevent fungal growth. Serial dilutions of the enriched broth were plated on nutrient agar media supplemented with 100 ppm and 300 ppm copper sulphate pentahydrate. Out of a total of 13 strains, 6 strains that were able to tolerate 300 ppm copper were selected for further study.

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Maximum tolerance capacity

Copper agar plates were prepared by incorporating different quantities of copper ranging from 10ppm to 100ppm solution of $CuSO_4$ into melted nutrient agar medium at 50˚C and poured into petridishes after mixing.

Bacterial cells were grown on nutrient agar slants for 24h at 37˚C and resuspended in sterilized distilled water at a concentration of approximately 10^8 CFU/ml. 100 µl of bacterial suspension was inoculated on copper agar plates with a micropipette. The strains that were able to grow in the maximum concentration of copper were subcultured again in nutrient agar plates having successive higher concentrations of copper of 100, 200, 300 etc up to the concentration after which the organism can't grow any further.

Resistance to other heavy metals

Resistance of the isolated bacteria to other important heavy metals such as Mercury, nickel, cadmium, cobalt and lead were also checked and their maximum tolerable capacity was found.

Identification of potential strain for copper tolerance

The potential strain was isolated and was identified biochemically up to species level following Bergey's manual of Determinative bacteriology and the identified strain was stored in nutrient agar slants for further study. Molecular level identification was done based on 16SrRNA sequencing results.

Copper accumulation in bacterial cell

Copper accumulation and adhesion was determined at various concentrations using ICP-OES technique. ICP process was done separately for supernatant and pellets to estimate the extracellular and intracellular compartmentalized copper. 24hrs culture broth was harvested by centrifugation at 6000 g for 10mins. To the supernatant 0.2N HNO_3 (1:1) was added and left for overnight incubation and checked for the amount of copper using ICP-OES. This value indicates the reduction in the total copper that is present in the media supernatant after the incubation period or after accumulation by the bacteria.

Optimization for effective copper accumulation

The shake-flask culture of the potential strain was further subjected to evaluate the effect of different environmental parameters like temperature, salinity,

pH and as well as different carbon sources on cell growth and copper toleration with slight modifications of Van-Thuoc *et al.* (2007).

Potential strain was tested against different pH (5.0 - 12.0) and temperatures (4^oC - 50^oC), salinity (0 - 30%), carbon sources, nitrogen sources and different copper concentrations. The cells were cultivated on a shaker (150 g) under aerobic condition. Growth, copper accumulation and toleration were determined at the end of cultivation (72 h).

Mass scale production

The potential was cultivated with all the optimized parameters in 1000ml conical flask with the optimized culture conditions in a shaker at 150g for 48hrs.

SEM Examination of copper accumulated bacterial Cells

For SEM analysis, samples of potential strain was fixed in 2.5% glutaraldehyde Millonig buffer phosphate for 2 h and washed four times in the same buffer. They were then dehydrated in successively increasing gradient concentrations of acetone (30, 50, 70, 90, and 100%) and dried by critical-point drying. Finally, all samples were mounted on metal stubs and coated with gold. A Jeol JSM-6300 scanning electron microscope (Jeol, Tokyo, Japan) was used to view the images.

Plasmid curing

Plasmid DNA of bacterial isolates was isolated according to Holmes (1984). Plasmid curing was done according to Lakshmi *et al.* (1988) and Crosa *et al.* (1994) using ethidiumbromide (100-600 mg/L). The colonies isolated from the ethidium bromide treatment were grown in nutrient agar medium containing 100 mg/L Cu^{2+} using grid pattern replica plating (Ohman 1988). The resistance of cured cultures was tested against copper agar plates. The cured cultures were spread on nutrient agar plates containing different concentrations of copper and incubated (37˚C) to estimate bacterial growth.

Results

Bacteria can resist metal stress due to the presence of cellular mechanisms of combating its toxic effects. They include deposition of toxic materials on cell walls, appropriate gene amplification, enhanced transcription of metallothionein genes, and alteration in the cell wall and plasma membrane complex.

Isolation of copper resistant bacteria

A number of bacterial strains showed significantly high levels of resistance to copper sulphate. These copper resistant strains were found in various genera tested, but particularly, the most potential strain named as WB1 for study purposes was specifically notable in its high resistance to copper. 13 initial strains were obtained in 100ppm of copper. Out of these 13 strains 6 potential strains were able to grow in 500ppm copper (Fig 1). Only 1 strain grew in 600ppm copper. This maximum tolerance capacity of the isolated strain (WB1) was found to be extremely high and hence assumed to have immense copper remediating property which was further subjected to experimental study.

Fig.1 Potential copper tolerating strains isolated

Resistance to other heavy metals

The resistance of WB1 to other important toxic heavy metals such as mercury, nickel, cadmium, cobalt, chromium and lead were also studied. The study indicated that WB1 was tolerant to a minimum level of 100ppm to the metals, cadmium and cobalt. The bacteria did not show any indications of growth in the presence of mercury while it showed growth upto 500ppm in the presence of both nickel and lead, 400 ppm for chromium. So, it can be concluded from this study that WB1 is resistant to higher level of copper than any other metal. Hence the importance of copper bioremediation in this study.

Identification of potential strain for copper tolerance

Biochemical identification of WB1 was done and found out to be *Bacillus cereus*.

Identification of potential strain by 16s rRNA sequencing

The potential strain WB1 was identified to be *Bacillus cereus* by 16SrRNA sequencing and the sequence is yet to be submitted to genbank. The phylogenetic tree was generated using Mega 4 software for the sequence obtained (Fig 2).

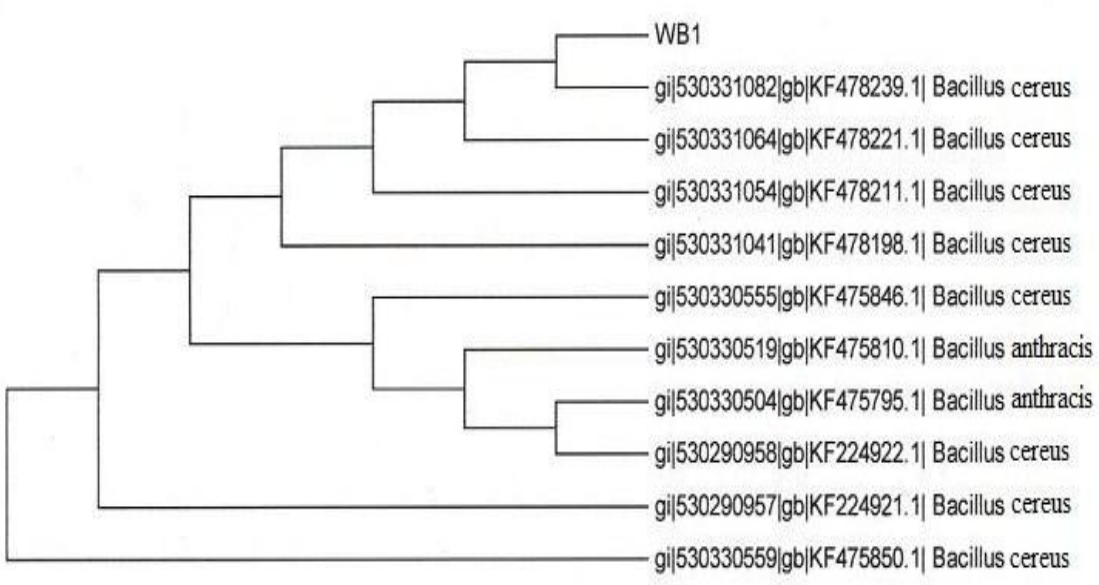


Fig. 2 Phylogenetic tree confirming the potential bacteria as *Bacillus cereus*

Optimization for effective copper accumulation

In the present investigation a series of optimized criteria was necessary for higher level of copper tolerance or accumulation by the bacteria. To study this, several physico-chemical parameters such as pH, temperature, salinity, concentraion were analysed. The cultures were maintained at various above mentioned criteria and then were analysed for copper concentration using ICP after following the necessary procedures.

The study also revealed that higher the cell biomass, higher is the copper accumulation. The bacterial growth at various concentrations of copper was analysed by taking OD at 600nm in UV-Visual Spectrophotometer and also by centrifuging the culture and weighing the biomass. High biomass and bacterial growth was obtained in 400ppm of copper addition to the culture. Hence a concentration of 400 ppm copper was maintained for all optimization tests.

In the growth kinetics study carried out, there was a gradual increase in cell mass as is evident by increase in cell dry weight of 0.189g at 12h to 0.405g at 24h per liter of the culture. However, a maximum accumulation of 48% (copper) of cell dry weight was obtained in 48h of incubation (Table 1).

Table. 1 Dry cell mass and copper accumulation at various incubation periods

S. No.	Species	Incubation period	Dry cell mass (g)	Copper accumulation(%)
1.	*Bacillus cereus*	12	0.589	11
2.		24	0.805	22
3.		36	1.721	39
4.		48	2.182	48
5.		60	1.872	17
6.		72	0.623	23

The optimum pH for *Bacillus cereus* was found to be 6 with a maximum accumulation of 45% (Fig 3).

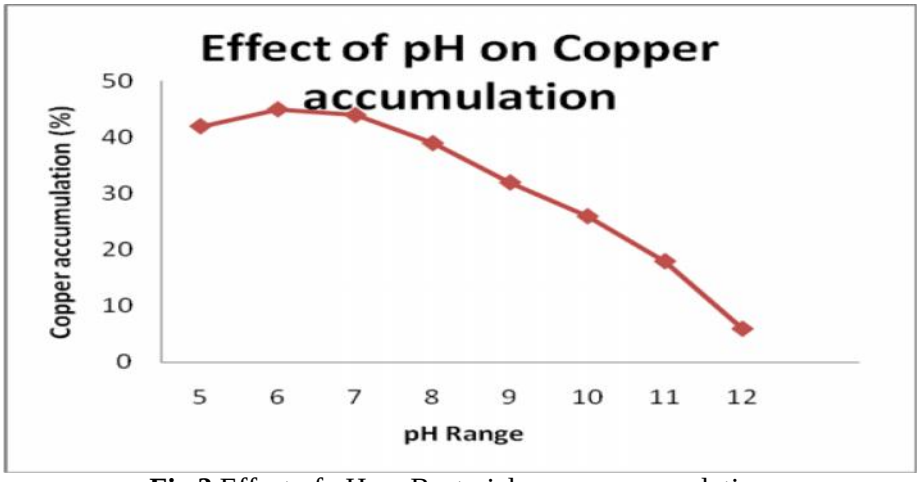


Fig.3 Effect of pH on Bacterial copper accumulation

The suitable temperature for copper accumulation was also verified by incubating the culture in the presence of copper at various temperatures and the optimum temperatures for *Bacillus cereus* was found to be 40˚C with a maximum accumulation of 45% (Fig 4).

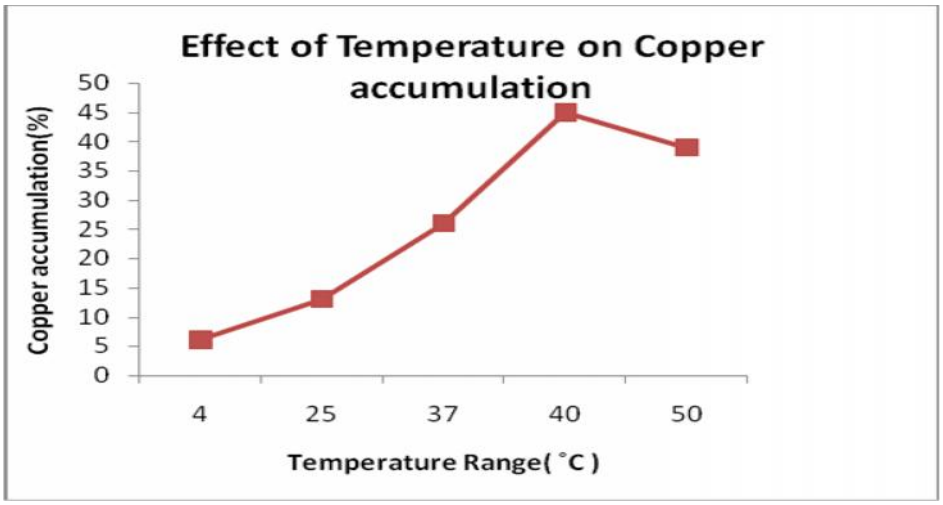


Fig.4 Effect of Temperature on Copper accumulation

In the present investigation salinity showed some influence on the copper accumulation and its maximum was recorded with 46% accumulation at 10% salinity in the case of *Bacillus cereus* (Fig 5).

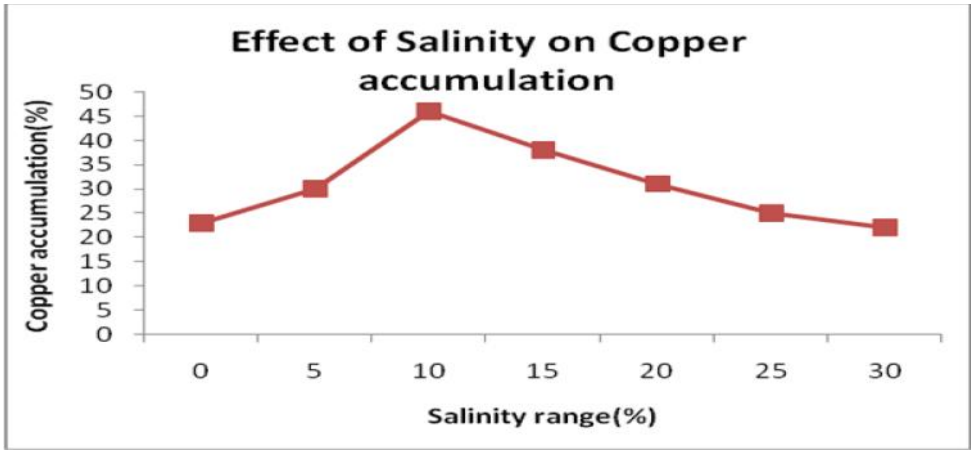


Fig.5 Effect of salinity on Copper accumulation

Among the different carbon sources tried copper accumulation was recorded highest when glucose was used as the sole carbon source (copper content of 42%) (Fig 6).

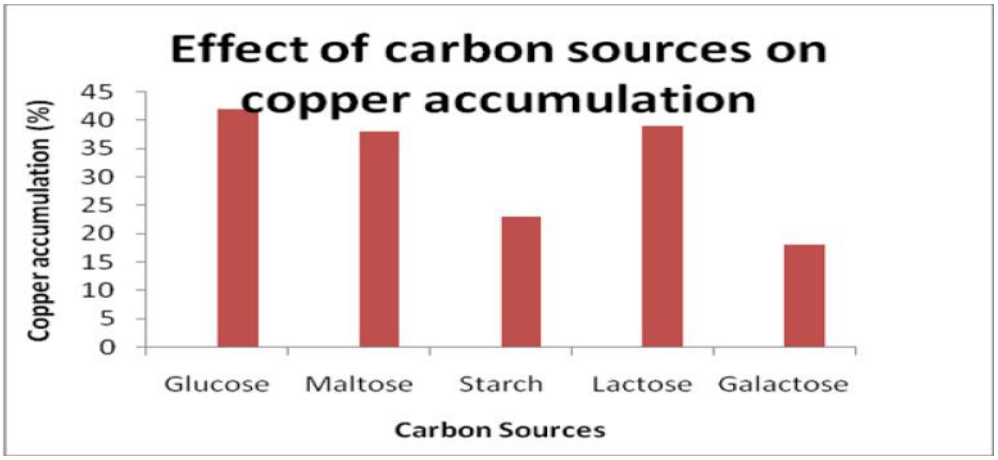


Fig.6 Effect of various carbon sources on copper accumulation

In the present study growth was influenced by the nitrogen source and in the presence of beef extract *Bacillus cereus* showed the maximum copper accumulation to about 48% (Fig 7).

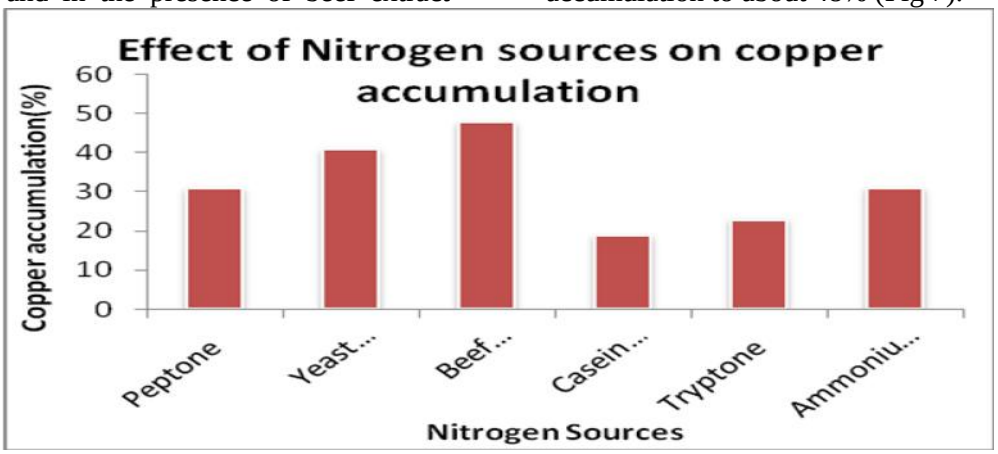


Fig.7 Effect of various nitrogen sources on copper accumulation

Mass scale production

After optimizing the culture conditions, mass scale culture of the most potential strain of *Bacillus cereus* was carried out with the obtained optimized readings. From the mass scale culture a maximum copper accumulation of 49% was obtained.

Plasmid curing

The location of the copper resistant gene whether in chromosomal DNA or plasmid DNA was found out by plasmid curing experiments by adding ethidium bromide at various concentrations to the culture. The culture were then plated with different concentrations of copper and their growth ability was noted. The colonies were able to grow even after curing them off plasmids and hence proving it to be the property of chromosomal DNA. Agarose gel electrophoresis of the plasmid DNA isolated also confirmed the absence of plasmids in the cured culture (Fig 8).

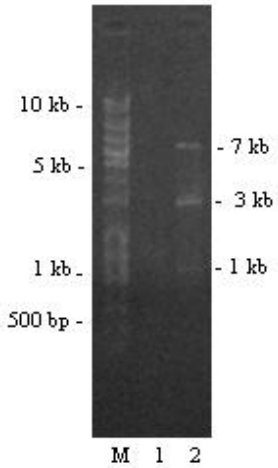
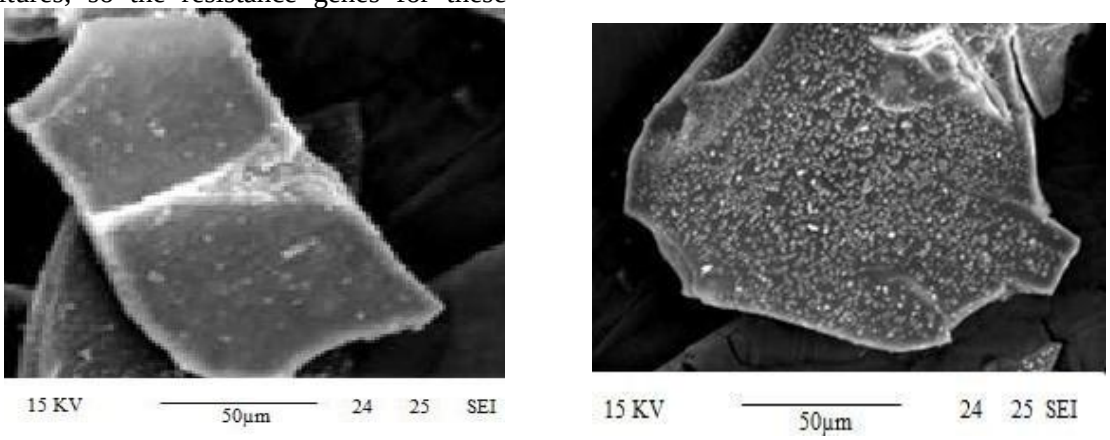


Fig.8 Plasmid profiling: Lane - M: 1kb DNA ladder, Lane -1 showing absence of plasmid (after curing) and Lane -2 showing plasmid pattern before curing in the *B. cereus*.

Resistance of cured and uncured culture of Cu^{2+} resistant bacterial isolates was also determined against other heavy metals. The resistance of cured cultures against Mercury, Cadmium and cobalt was significantly lower compared with uncured cultures; this indicates that the gene for resistance against these metals is located on a chromosome and/or plasmids. The resistance of cured cultures against nickel and lead was not significantly lower than that of the uncured cultures; so the resistance genes for these metals are considered to be located on plasmids as well as on chromosomes. As the cured cultures of the isolates showed a higher resistance against Pb^{2+} than the uncured cultures, the gene for Pb^{2+} is probably located on the chromosomes.

SEM analysis revealed striking results. Clear deposition of copper over the cell wall of dried biomass was observed in copper treated cells. The control was devoid of these depositions (Fig 9).



(a) Control (b) Copper treated

Fig. 9 SEM image showing copper adhesion

Discussion

Bacteria can resist metal stress due to the presence of cellular mechanisms of combating its toxic effects; they include, deposition of toxic material on cell walls (Brown and Smith 1976), appropiate gene amplification (Beach and Palmiter 1981), enhanced transcription of metallothionein genes (Hildebrand *et al.* 1982) and alteration in the cell wall and plasma membrane complex (Grindle 1984). Some of these terms were taken into account and hence the study.

Accumulation of metals by all microorganisms depends on external pH and decreases with lower pH (Morley and Gadd 1995) also extreme temperature is usually a limiting factor. Usually, AAS was used in determining the copper level or metal level in a bacterial cell. ICP is considered to be a higher version of AAS and hence ICP study is used for the measurement of copper level absorbed and adsorbed by the bacterial cell in this study.
On analyzing the present study it was clearly observed that *Bacillus cereus* isolated from the copper contaminated industrial coast with a maximum tolerable capacity of 600ppm which is significantly higher than most reported tolerance level can be considered a highly potential strain for bacterial bioremediation of contaminated area.

Khusro A *et al.* (2014) reported on the multiple heavy metals response of *Bacillus subtilis* strain KPA and observed a maximum tolerable capacity of *Bacillus subtilis* strain KPA against multiple heavy metals. The strain was able to tolerate Cr^{6+} and Cu^{2+} at the maximum concentration of 200 mg/l and 800 mg/l respectively. The above study shows the multiple heavy metal resistance of the Bacillus spp. similarly in the present study, *Bacillus cereus* shows multiple heavy metal resistance against nickel, lead and cobalt, however the strain was susceptible to mercury and cadmium as these metals are considered extremely toxic even at a very low concentration. In the present study, the isolated strain of *Bacillus cereus* showed resistance against multiple heavy metals. The strain was studied for its resistance against nickel, lead, cobalt, chromium, cadmium and mercury. The strain showed a MTC (Maximum tolerable capacity) of 100ppm against cobalt and cadmium. 400 ppm against chromium, 500 ppm growth in the presence of nickel and lead respectively making it a very potential organism for heavy metal remediation. The organism was highly susceptible to mercury as it is the most toxic heavy metal.

Bioremediation of copper onto the surface of a microorganism is affected by several factors such as initial pH, initial copper ion concentration, time and temperature etc. The level of the three variables: pH 6.18; initial copper concentration, 32.50 mg L^{-1} , time 30 hours, were found to be optimum for maximum copper removal. The corresponding removal in optimum condition was found to be 60.264% as experimemted by Arpita Ghosh *et al.* (2013). An increase in copper concentration had a negative impact on biosorption efficiency (Das *et al.* 2008).

Hossian and Anantharaman (2005) also investigated the effect of temperature on copper biosorption and it was found that increasing temperature increases copper biosorption up to 40°C, beyond which biosorption decreases. Salinity was checked as the bacteria was isolated from the coastal area. Finally, with all the above optimization parameters provided a total accumulation of 49% of copper was obtained, making it a very suitable candidate for use in copper bioremediation. The percentage accumulation is found to be on par with the accumulation ranges of other organisms.

In the present study, many optimization parameters such as pH, temperature, salinity, copper concentration and also the nutritional requirements of the bacteria such as carbon sources and nitrogen sources were also studied. In this way all the environmental conditions of the bacteria was effectively conditioned and monitored for its efficient growth and copper accumulation. The maintained values were pH 6 , temperature 40˚C, salinity 10%, copper concentration 400 ppm, carbon source glucose, nitrogen source beef extract.

Plasmid elimination is considered to take place in ecosystems containing numerous bacterial species. This opens up a new perspective in bioremediation and rational drug design. Resistance to toxic metal ions in bacteria had been reported to be associated with plasmids (Mergeay 1991). Bacterial plasmids encode a resistance system for toxic metals including Ag^{+}, As^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+} and Pb^{2+} (Silver and Phung 1996). Chromosome-encoded Cu^{2+} resistance was described by Silver (1996), moreover, a chromosomal locus required for Cu^{2+} resistance and

competitive fitness was cloned from a strain of *Pseudomonas fluorescens* isolated from copper-contaminated agriculture soil (Hong *et al.* 1996). In *Pseudomonas syringae* the gene for copper and streptomycin is located on a plasmid (Sundin and Bender 1996) whereas in this particular study, the gene for copper tolerance is absent in the plasmid as the result of plasmid curing experiments and hence it is assumed to be present in the chromosomal DNA.
Based on the studies reported, in the present research, location of the copper resistance gene in plasmid or chromosome was analysed using Ethidium Bromide as the curing agent. The cured cultures were tested for copper resistance by plating it in nutrient agar plates added with copper. The colonies were able to grow even after plasmid curing indicating the presence of copper resistant gene in the chromosomal DNA.

Mamba *et al.* (2009) studied the interactions of the micro-organisms with the metal species using Scanning Electron Microscopy (SEM). The SEM micrograph showed the accumulation of metal ions on the cell wall of the bacterial species. The TEM micrograph confirmed the SEM micrograph showing presence of dark accumulations in the cell of the bacteria.

In the present study, the dry cells of bacteria were analyzed for its copper absorption using SEM analysis. There was a vast difference between the control cells without copper and copper treated cells. The copper treated SEM micrograph showed tiny adhesions that appear bright when compared to other cells in control. This might be because of the deposition of copper onto the cell wall of the bacteria making it bright visibly. Similar results were also observed by Zapotoczny *et al.* (2007) when he examined copper loaded fungi which showed dense deposition in its mycelium.

Our bacterial strains have an efficient Cu^{2+} accumulating system, which make possible their use in environmental bioremediation. The development of a microbiological detoxification strategy leads to a better exploitation of bacteria in the treatment of wastewater released by industry (Bender *et al.* 1995). There are other physical and biochemical procedures for the detoxification of metal wastewaters but microbiological detoxification of polluted water is recommended as economical and safe, and involves less negative secondary effects on the environment.

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